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(54) Title: ALPHAVIRUS RNA REPLICON SYSTEMS

(57) Abstract

The present invention provides a helper cell for expressing an infectious, replication defective, alphavirus particle in an alphavirus-permissive cell. The helper cell includes (a) a first helper RNA encoding (i) at least one alphavirus structural protein, and (ii) not encoding at least one alphavirus structural protein; and (b) a second helper RNA separate from the first helper RNA, the second helper RNA (i) not encoding the alphavirus structural protein encoded by the first helper RNA, and (ii) encoding the at least one alphavirus structural protein not encoded by the first helper RNA. Preferably, the helper cell is co-transfected with a replicon RNA encoding an alphavirus packaging segment and an inserted heterogeneous RNA, such that all of the alphavirus structural proteins assemble together into alphavirus particles in the cell, with said replicon RNA packaged therein.

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ALPHAVIRUS RNA REPLICON SYSTEMS

Field of the Invention

The present invention relates to recombinant DNA technology, and in particular to introducing and expressing foreign genes in a eukaryotic cell.

Background of the Invention

The Alphavirus genus includes a variety of viruses all of which are members of the Togaviridae family. The alphaviruses include Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Everglades virus, Mucambo virus, Pixuna virus, Western Equine Encephalitis virus (WEE), Sindbis virus, Semliki Forest virus, Middelburg virus, Chikungunya virus, O'nyong-nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, and Buggy Creek virus. The viral genome is a single-stranded, messenger-sense RNA, modified at the 5'-end with a methylated cap, and at the 3'-end with a variable-length poly (A) tract. Structural subunits containing a single viral protein, C, associate with the RNA genome in an icosahedral nucleocapsid.

In the virion, the capsid is surrounded by a lipid envelope covered with a regular array of transmembranal protein spikes, each of which consists of a heterodimeric complex of two glycoproteins, E1 and E2. *See* Pedersen et al., *J. Virol.* **14**:40 (1974). The Sindbis and Semliki Forest viruses are considered the prototypical alphaviruses, and have been studied extensively. *See* Schlesinger, *The Togaviridae and Flaviviridae*, Plenum Publishing Corp., New York (1986). The VEE virus has been studied by the present inventors. *See* U.S. Patent No. 5,185,440 to Davis et al.

The study of these viruses has led to the development of beneficial techniques for vaccinating against the alphavirus diseases, and other diseases through the use of alphavirus vectors for the introduction of foreign DNA. *See* U.S. Patent No. 5,185,440 to Davis et al., and PCT Publication WO 92/10578. The introduction of foreign DNA into eukaryotic cells has become a topic of increasing interest. It is well known that live, attenuated viral vaccines are among the most successful means of controlling viral disease. However, for some virus pathogens, immunization with a live virus strain may be either impractical or unsafe. One alternative strategy is the insertion of sequences encoding immunizing antigens of such agents into a vaccine strain of another virus. One such system utilizing a live VEE vector is described in our copending Patent Application Serial No. 08/250,445, filed 27 May 1994. Another such system is described by Hahn et al., *Proc. Natl. Acad. Sci. USA* **89**:2679 (1992), wherein Sindbis virus constructs which express a truncated form of the influenza hemagglutinin protein are described. Unfortunately, relatively few such systems are currently available.

Accordingly, there remains a need in the art for nucleic acid sequences encoding foreign antigens to be safely incorporated into a vaccine strain of a virus, which may be then be utilized as a vaccine for the foreign antigen.

Summary of the Invention

As a first aspect, the present invention provides a helper cell for expressing an infectious, replication defective, alphavirus particle in an alphavirus-permissive cell. The helper cell includes (a) a first helper RNA encoding *(i)* at least one alphavirus structural protein, and *(ii)* not encoding at least one alphavirus

structural protein; and (b) a second helper RNA separate from the first helper RNA, the second helper RNA *(i)* not encoding the at least one alphavirus structural protein encoded by the first helper RNA, and *(ii)* encoding the at least one alphavirus structural protein not encoded by the first helper RNA, such that the alphavirus structural proteins assemble together into alphavirus particles in the cell. Preferably, the alphavirus packaging segment is deleted from at least the first helper RNA, and is more preferably deleted from both the first helper RNA and second helper RNA.

In a preferred embodiment, the helper cell is co-transfected with a replicon RNA, which encodes the alphavirus packaging segment and an inserted heterologous RNA. In the embodiment wherein the helper cell also includes a replicon RNA, the alphavirus packaging segment may be, and preferably is, deleted from both the first helper RNA and the second helper RNA. For example, in the embodiment wherein the helper cell includes a replicon RNA encoding the alphavirus packaging segment and an inserted heterologous RNA, the first helper RNA encodes the alphavirus El glycoprotein and the alphavirus E2 glycoprotein, and the second helper RNA encodes the alphavirus capsid protein. The replicon RNA, first helper RNA, and second helper RNA are all on separate molecules and are co-transfected into the host cell.

In an alternative embodiment, the helper cell includes a first helper RNA encoding the alphavirus El glycoprotein and the alphavirus E2 glycoprotein, and is co-transfected with a replicon RNA encoding the alphavirus packaging segment, an inserted heterologous RNA, and the remaining alphavirus structural proteins not encoded by a first helper RNA. Thus, the replicon RNA and the first helper RNA are on separate molecules, and the replicon RNA and the RNA encoding a structural protein not encoded by the first helper RNA are on a single molecule. The heterologous RNA comprises a foreign RNA which encodes for proteins or peptides which are desirably expressed in the helper cell.

The RNA encoding the structural proteins, i.e., the first helper RNA and the second helper RNA, may advantageously include one or more attenuating mutations. In the preferred embodiment, at least one of the first helper RNA and the second helper RNA includes at least one attenuating mutation. The attenuating

mutations provide the advantage that in the event of RNA recombination within the cell, the conjoining of the structural and non-structural genes will produce a virus of decreased virulence.

As a second aspect, the present invention provides a method of making infectious, replication defective, alphavirus particles. The method includes co-transfecting a helper cell as given above with a replicon RNA, producing the alphavirus particles in the transfected cell, and then collecting the alphavirus particles from the cell. The replicon RNA encodes the alphavirus packaging segment, non-structural proteins and a heterologous RNA. The non-structural proteins encoded by the replicon RNA may be such proteins as are required for replication and transcription. The transfected cell further includes the first helper RNA and second helper RNA as described above.

As a third aspect, the present invention provides a set of RNAs for expressing an infectious, replication defective alphavirus. The set of RNAs comprises, in combination, (a) a replicon RNA encoding a promoter sequence, an inserted heterologous RNA, wherein RNA encoding at least one structural protein of the alphavirus is deleted from the replicon RNA, and (b) a first helper RNA separate from the replicon RNA, wherein the first helper RNA encodes *in trans*, the structural protein which is deleted from the replicon RNA, and a promoter sequence. In this embodiment, it is preferred that an RNA segment encoding at least one of the structural proteins is located on an RNA other than the first helper RNA. Thus, for example, the set of RNAs may include a replicon RNA including RNA which encodes the alphavirus packaging sequence, the inserted heterologous RNA, and the alphavirus capsid protein, but both the alphavirus E1 glycoprotein and alphavirus E2 glycoprotein are deleted therefrom; and a first helper RNA includes RNA encoding both the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein.

In another embodiment, the set of RNAs also includes a second helper RNA separate from the replicon RNA and the first helper RNA. In this embodiment, the second helper RNA encodes, *in trans*, at least one structural protein, which is different from the structural protein encoded by the replicon RNA and by the first helper RNA. Thus, for example, the set of RNAs may

include a replicon RNA including RNA which encodes the alphavirus packaging sequence, and the inserted heterologous RNA; a first helper RNA including RNA which encodes a promoter sequence and an RNA encoding both the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein; and a second helper RNA including a promoter sequence and RNA which encodes the alphavirus capsid protein, with the replicon RNA, the first helper RNA, and the second helper RNA being *in trans* from each other, on separate molecules.

As a fourth aspect, the present invention provides infectious VEE replicon particles containing RNA encoding a promoter sequence, and an inserted heterologous RNA, and wherein RNA encoding at least one alphavirus structural protein is deleted from the RNA so that the infectious virus particle is replication defective.

As a fifth aspect, the present invention provides a pharmaceutical formulation comprising infectious alphavirus particles as described above, in an effective immunogenic amount in a pharmaceutically acceptable carrier.

The foregoing and other aspects of the present invention are explained in detail in the detailed description set forth below.

Brief Description of the Drawings

Figure 1 is a graphical representation of the pV4031 clone and the production of the pVR2 clone.

Figure 2 illustrates the construction of the double helper RNA system plasmids in accordance with the present invention. In the drawings designated P21-1 and P24-3, the dashed lines indicate structural proteins or portions thereof which are deleted in the plasmid. **Figure 2** also illustrates a single helper RNA system plasmid, and the construction of recombinant VEE clones containing heterologous genes.

Figure 3 is a graphical representation of the results obtained in inoculating mice with the VEE replicon/Lassa N infectious particles produced by a single-helper RNA system, at two different dosage units. The top graph represents results obtained with a low-dose inoculation with infectious particles.

The bottom graph illustrates results obtained with a high-dose inoculation with infectious particles.

Detailed Description of the Invention

The term "alphavirus" has its conventional meaning in the art, and includes the various species of alphaviruses such as Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Everglades virus, Mucambo virus, Pixuna virus, Western Equine Encephalitis virus (WEE), Sindbis virus, Semliki Forest virus, Middelburg virus, Chikungunya virus, O'nyong-nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, Buggy Creek virus, and any other virus classified by the International Committee on Taxonomy of Viruses (ICTV) as an alphavirus. The preferred alphavirus RNA transcripts for use in the present invention include VEE, Sindbis virus, and Semliki Forest virus.

Alphavirus-permissive cells employed in the methods of the present invention are cells which, upon transfection with the viral RNA transcript, are capable of producing viral particles. Alphaviruses have a broad host range. Examples of suitable host cells include, but are not limited to Vero cells, baby hamster kidney (BHK) cells, and chicken embryo fibroblast cells.

The phrases "structural protein" or "alphavirus structural protein" as used herein refer to the encoded proteins which are required for encapsidation (e.g., packaging) of the RNA replicon, and include the capsid protein, E1 glycoprotein, and E2 glycoprotein. As described hereinabove, the structural proteins of the alphavirus are distributed among one or more helper RNAs (i.e., a first helper RNA and a second helper RNA). In addition, one or more structural proteins may be located on the same RNA molecule as the replicon RNA, provided that at least one structural protein is deleted from the replicon RNA such that the resulting alphavirus particle is replication defective. As used herein, the terms "deleted" or "deletion" mean either total deletion of the specified segment or the deletion of a sufficient portion of the specified segment to render the segment

inoperative or nonfunctional, in accordance with standard usage. *See, e.g.*, U.S. Patent No. 4,650,764 to Temin et al. The term "replication defective" as used herein, means that the replicon RNA cannot be encapsidated in the host cell in the absence of the helper RNA. The resulting alphavirus particles are replication defective inasmuch as the replicon RNA does not include all of the alphavirus structural proteins required for encapsidation, at least one of the required structural proteins being deleted therefrom, such that the packaged replicon RNA is not capable of replicating the entire viral genome.

The helper cell for expressing the infectious, replication defective alphavirus particle comprises a set of RNAs, as described above. The set of RNAs principally include a first helper RNA and a second helper RNA. The first helper RNA includes RNA encoding at least one alphavirus structural protein but does not encode all alphavirus structural proteins. In other words, the first helper RNA does not encode at least one alphavirus structural protein; the at least one non-coded alphavirus structural protein being deleted from the first helper RNA. In one embodiment, the first helper RNA includes RNA encoding the alphavirus E1 glycoprotein, with the alphavirus capsid protein and the alphavirus E2 glycoprotein being deleted from the first helper RNA. In another embodiment, the first helper RNA includes RNA encoding the alphavirus E2 glycoprotein, with the alphavirus capsid protein and the alphavirus E1 glycoprotein being deleted from the first helper RNA. In a third, preferred embodiment, the first helper RNA includes RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, with the alphavirus capsid protein being deleted from the first helper RNA.

The second helper RNA includes RNA encoding at least one alphavirus structural protein which is different from the at least one structural protein encoded by the first helper RNA. Thus, the second helper RNA encodes at least one alphavirus structural protein which is not encoded by the first helper RNA. The second helper RNA does not encode the at least one alphavirus structural protein which is encoded by the first helper RNA, thus the first and second helper RNAs do not encode duplicate structural proteins. In the embodiment wherein the first helper RNA includes RNA encoding only the

alphavirus El glycoprotein, the second helper RNA may include RNA encoding one or both of the alphavirus capsid protein and the alphavirus E2 glycoprotein which are deleted from the first helper RNA. In the embodiment wherein, the first helper RNA includes RNA encoding only the alphavirus E2 glycoprotein, the second helper RNA may include RNA encoding one or both of the alphavirus capsid protein and the alphavirus El glycoprotein which are deleted from the first helper RNA. In the embodiment wherein the first helper RNA includes RNA encoding both the alphavirus El glycoprotein and the alphavirus E2 glycoprotein, the second helper RNA may include RNA encoding the alphavirus capsid protein which is deleted from the first helper RNA.

In one embodiment, the packaging segment (RNA comprising the encapsidation or packaging signal) is deleted from at least the first helper RNA. In a preferred embodiment, the packaging segment is deleted from both the first helper RNA and the second helper RNA.

In the preferred embodiment wherein the packaging segment is deleted from both the first helper RNA and the second helper RNA, the helper cell is co-transfected with a replicon RNA in addition to the first helper RNA and the second helper RNA. The replicon RNA encodes the packaging segment and an inserted heterologous RNA. The inserted heterologous RNA may be RNA encoding a protein or a peptide. Typically, the inserted heterologous RNA encodes a protein or a peptide which is desirably expressed by the host alphavirus-permissive cell, and includes the promoter and regulatory segments necessary for the expression of that protein or peptide in that cell. The inserted heterologous RNA may encode any protein or peptide that may be desirably produced by the host cell. Suitable heterologous RNA may be of prokaryotic (e.g., RNA encoding the *Botulinus* toxin C), or eukaryotic (e.g., RNA from *Aqueoria victoria* jellyfish encoding the green fluorescent protein (GFP), RNA encoding malaria *Plasmodium* protein cs1) origin.

Additionally, inserted heterologous RNA suitable in the practice of the present invention include viral RNA from a wide variety of viruses including, but not limited to, Arenaviruses (e.g., Lassa fever virus), Lentiviruses (e.g., HIV, SIV, Equine infectious anemia virus), Poxviruses (e.g., Vaccinia), Filoviruses

(e.g., Ebola virus, Marburg virus), Orthomyxoviruses (e.g., Influenza virus), Bunyaviruses (e.g., RVFV, CCHF, and SFS viruses), and Coronaviruses. Examples of suitable viral RNA genes that may be used to provide the inserted heterologous RNA include, but are not limited to the Lassa fever virus nucleocapsid protein gene, the Lassa fever envelope glycoprotein gene, the influenza hemagglutinin gene, the influenza nucleoprotein gene, the human coronavirus envelope glycoprotein gene, the HIV envelope GP160 gene, and the HIV matrix/capsid gene. The replicon RNA also encodes the alphavirus nonstructural proteins, including *cis*-acting sequences required for replication and transcription.

In a preferred embodiment, the replicon RNA, the first helper RNA and the second helper RNA are provided on separate molecules such that a first molecule, i.e., the replicon RNA, includes RNA encoding the packaging segment and the inserted heterologous RNA, a second molecule, i.e., the first helper RNA, includes RNA encoding at least one but not all of the required alphavirus structural proteins, and a third molecule, i.e., the second helper RNA, includes RNA encoding at least one but not all of the required alphavirus structural proteins. For example, in one preferred embodiment of the present invention, the helper cell includes a set of RNAs which include (a) a replicon RNA including RNA encoding an alphavirus packaging sequence and an inserted heterologous RNA, (b) a first helper RNA including RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, and (c) a second helper RNA including RNA encoding the alphavirus capsid protein so that the alphavirus E1 glycoprotein, the alphavirus E2 glycoprotein and the capsid protein assemble together into alphavirus particles in the host cell.

In an alternate embodiment, the replicon RNA and the first helper RNA are on separate molecules, and the replicon RNA and RNA encoding a structural gene not encoded by the first helper RNA are on another single molecule together, such that a first molecule, i.e., the first helper RNA, including RNA encoding at least one but not all of the required alphavirus structural proteins, and a second molecule, i.e., the replicon RNA, including RNA encoding the packaging segment, the inserted heterologous RNA, and the remaining structural proteins not

encoded by the first helper RNA. For example, in one preferred embodiment of the present invention, the helper cell includes a set of RNAs including (a) a replicon RNA including RNA encoding an alphavirus packaging sequence, an inserted heterologous RNA, and an alphavirus capsid protein, and (b) a first helper RNA including RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein so that the alphavirus E1 glycoprotein, the alphavirus E2 glycoprotein and the capsid protein assemble together into alphavirus particles in the host cell, with the replicon RNA packaged therein.

In one preferred embodiment of the present invention, the RNA encoding the alphavirus structural proteins, i.e., the capsid, E1 glycoprotein and E2 glycoprotein, contains at least one attenuating mutation. The phrases "attenuating mutation" and "attenuating amino acid," as used herein, mean a nucleotide sequence containing a mutation, or an amino acid encoded by a nucleotide sequence containing a mutation, which mutation results in a decreased probability of causing disease in its host (i.e., a loss of virulence), in accordance with standard terminology in the art, *See, e.g.,* B. Davis, et al., Microbiology 132 (3d ed. 1980), whether the mutation be a substitution mutation or an in-frame deletion mutation. The phrase "attenuating mutation" excludes mutations or combinations of mutations which would be lethal to the virus. Thus, according to this embodiment, at least one of the first helper RNA and the second helper RNA includes at least one attenuating mutation. In a more preferred embodiment, at least one of the first helper RNA and the second helper RNA includes at least two, or multiple, attenuating mutations. The multiple attenuating mutations may be positioned in either the first helper RNA or in the second helper RNA, or they may be distributed randomly with one or more attenuating mutations being positioned in the first helper RNA and one or more attenuating mutations positioned in the second helper RNA. Alternatively, when the replicon RNA and the RNA encoding the structural proteins not encoded by the first helper RNA are located on the same molecule, an attenuating mutation may be positioned in the RNA which codes for the structural protein not encoded by the first helper RNA. The attenuating mutations may also be located within the RNA encoding non-structural proteins (e.g., the replicon RNA).

Appropriate attenuating mutations will be dependent upon the alphavirus used. For example, when the alphavirus is VEE, suitable attenuating mutations may be selected from the group consisting of codons at E2 amino acid position 76 which specify an attenuating amino acid, preferably lysine, arginine, or histidine as E2 amino acid 76; codons at E2 amino acid position 120 which specify an attenuating amino acid, preferably lysine as E2 amino acid 120; codons at E2 amino acid position 209 which specify an attenuating amino acid, preferably lysine, arginine, or histidine as E2 amino acid 209; codons at E1 amino acid 272 which specify an attenuating mutation, preferably threonine or serine as E1 amino acid 272; codons at E1 amino acid 81 which specify an attenuating mutation, preferably isoleucine or leucine as E1 amino acid 81; codons at E1 amino acid 253 which specify an attenuating mutation, preferably serine or threonine as E1 amino acid 253; and the combination mutation of the deletion of E3 codons 56-59 together with codons at E1 amino acid 253 which specify an attenuating mutation, as provided above. Other suitable attenuating mutations within the VEE genome will be known to those skilled in the art.

In an alternate embodiment, wherein the alphavirus is the South African Arbovirus No. 86 (S.A.AR86), suitable attenuating mutations are located on the RNA molecule encoding both non-structural and structural proteins, and may be selected from the group consisting of codons at nsP1 amino acid position 538 which specify an attenuating amino acid, preferably isoleucine as nsP1 amino acid 538; codons at E2 amino acid position 304 which specify an attenuating amino acid, preferably threonine as E2 amino acid 304; codons at E2 amino acid position 314 which specify an attenuating amino acid, preferably lysine as E2 amino acid 314; codons at E2 amino acid 372 which specify an attenuating amino acid, preferably valine, at E2 amino acid residue 372; codons at E2 amino acid position 376 which specify an attenuating amino acid, preferably alanine as E2 amino acid 376; in combination, codons at E2 amino acid residues 304, 314, 372, and 376 which specify attenuating amino acids at E2 amino acid residues 304, 314, 372, and 376; codons at E2 amino acid position 378 which specify an attenuating amino acid, preferably leucine as E2 amino acid 378; codons at nsP2 amino acid position 96 which specify an attenuating amino acid, preferably glycine as nsP2 amino acid

96; and codons at nsP2 amino acid position 372 which specify an attenuating amino acid, preferably valine as nsP2 amino acid 372; in combination, codons at nsP2 amino acid residues 96 and 372 attenuating substitution mutations at nsP2 amino acid residues 96 and 372; codons at nsP2 amino acid residue 529 which specify an attenuating amino acid, preferably leucine, at nsP2 amino acid residue 529; codons at nsP2 amino acid residue 571 which specify an attenuating amino acid, preferably asparagine, at nsP2 amino acid residue 571; codons at nsP2 amino acid residue 682 which specify an attenuating amino acid, preferably arginine, at nsP2 amino acid residue 682; codons at nsP2 amino acid residue 804 which specify an attenuating amino acid, preferably arginine, at nsP2 amino acid residue 804; codons at nsP3 amino acid residue 22 which specify an attenuating amino acid, preferably arginine, at nsP3 amino acid residue 22; and in combination, codons at nsP2 amino acid residues 529, 571, 682, and 804, and at nsP3 amino acid residue 22, specifying attenuating amino acids at nsP2 amino acid residues 529, 571, 682, and 804, and at nsP3 amino acid residue 22. Suitable attenuating mutations useful in embodiments wherein other alphaviruses are employed are known to those skilled in the art. Attenuating mutations may be introduced into the RNA by performing site-directed mutagenesis on the cDNA which encodes the RNA, in accordance with known procedures. *See*, Kunkel, *Proc. Natl. Acad. Sci. USA* **82**:488 (1985), the disclosure of which is incorporated herein by reference in its entirety. Alternatively, mutations may be introduced into the RNA by replacement of homologous restriction fragments in the cDNA which encodes for the RNA, in accordance with known procedures.

Preferably, the first helper RNA and the second helper RNA also include a promoter. It is also preferred that the replicon RNA also includes a promoter. Suitable promoters for inclusion in the first helper RNA, second helper RNA and replicon RNA are well known in the art. One preferred promoter is the VEE 26S promoter for use when the alphavirus is VEE. Additional promoters beyond VEE 26S include the Sindbis 26S promoter, the Semliki Forest 26S promoter, and any other promoter sequence recognized by alphavirus polymerases. Alphavirus promoter sequences containing mutations which alter the activity level of the promoter (in relation to the activity level of the wild-type) are also suitable

in the practice of the present invention. Such mutant promoter sequences are described in in Raju and Huang, *J. Virol.* 65, 2501-2510 (1991), the disclosure of which is incorporated herein in its entirety. In the system wherein the first helper RNA, the second helper RNA, and the replicon RNA are all on separate molecules, the promoters, if the same promoter is used for all three RNAs, provide a homologous sequence between the three molecules. It is preferred that the selected promoter is operative with the non-structural proteins encoded by the replicon RNA molecule.

In cases where vaccination with two immunogens provides improved protection against disease as compared to vaccination with only a single immunogen, a double-promoter replicon would ensure that both immunogens are produced in the same cell. Such a replicon would be the same as the one described above, except that it would contain two copies of the 26S RNA promoter, each followed by a different multiple cloning site, to allow for the insertion and expression of two different heterologous proteins. Another useful strategy is to insert the IRES sequence from the picornavirus, EMC virus, between the two heterologous genes downstream from the single 26S promoter of the replicon described above, thus leading to expression of two immunogens from the single replicon transcript in the same cell.

The infectious, replication defective alphavirus particles may be prepared according to the methods disclosed herein in combination with techniques known to those skilled in the art. The method includes transfecting an alphavirus-permissive cell with a replicon RNA including the alphavirus packaging segment and an inserted heterologous RNA, a first helper RNA including RNA encoding at least one alphavirus structural protein, and a second helper RNA including RNA encoding at least one alphavirus structural protein which is different from that encoded by the first helper RNA; producing the alphavirus particles in the transfected cell; and collecting the alphavirus particles from the cell. The step of transfecting the alphavirus-permissive cell can be carried out according to any suitable means known to those skilled in the art. For example, uptake of the RNA into the cells can be achieved by any suitable means, such as for example, by treating the cells with DEAE-dextran, treating the RNA with "LIPOFECTIN™"

before addition to the cells, or by electroporation, with electroporation being the currently preferred means of achieving RNA uptake into the alphavirus-permissive cells. These techniques are well known in the art. *See e.g.*, U.S. Patent No. 5,185,440 to Davis et al., and PCT Publication No. WO 92/10578 to Bioption AB, the disclosures of which are incorporated herein by reference in their entirety.

The step of facilitating the production of the infectious viral particles in the cells may also be carried out using conventional techniques. *See e.g.*, U.S. Patent No. 5,185,440 to Davis et al., PCT Publication No. WO 92/10578 to Bioption AB, and U.S. Patent No. 4,650,764 to Temin et al. (although Temin et al., relates to retroviruses rather than alphaviruses). The infectious viral particles may be produced by standard cell culture growth techniques.

The step of collecting the infectious alphavirus particles may also be carried out using conventional techniques. For example, the infectious particles may be collected by cell lysis, or collection of the supernatant of the cell culture, as is known in the art. *See e.g.*, U.S. Patent No. 5,185,440 to Davis et al., PCT Publication No. WO 92/10578 to Bioption AB, and U.S. Patent No. 4,650,764 to Temin et al. Other suitable techniques will be known to those skilled in the art. Optionally, the collected infectious alphavirus particles may be purified if desired. Suitable purification techniques are well known to those skilled in the art.

Pharmaceutical formulations, such as vaccines, of the present invention comprise an immunogenic amount of the infectious, replication defective alphavirus particles as disclosed herein in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the infectious alphavirus particles which is sufficient to evoke an immune response in the subject to which the pharmaceutical formulation is administered. An amount of from about 10^3 to about 10^7 replicon-containing particles, and preferably about 10^4 to 10^6 replicon-containing particles per dose is believed suitable, depending upon the age and species of the subject being treated, and the immunogen against which the immune response is desired. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution. Subjects which may be administered immunogenic amounts of the infectious, replication defective alphavirus particles of the present

invention include but are not limited to human and animal (e.g., pig, cattle, dog, horse, donkey, mouse, hamster, monkeys) subjects.

Pharmaceutical formulations of the present invention include those suitable for parenteral (e.g., subcutaneous, intradermal, intramuscular, intravenous and intraarticular) administration. Alternatively, pharmaceutical formulations of the present invention may be suitable for administration to the mucus membranes of a subject (e.g., intranasal administration). The formulations may be conveniently prepared in unit dosage form and may be prepared by any of the methods well known in the art.

The helper cells, RNAs and methods of the present invention are useful in *in vitro* expression systems, wherein the inserted heterologous RNA located on the replicon RNA encodes a protein or peptide which is desirably produced *in vitro*. The helper cells, RNAs, methods and pharmaceutical formulations of the present invention are additionally useful in a method of administering a protein or peptide to a subject in need of the desired protein or peptide, as a method of treatment or otherwise. In this embodiment of the invention, the heterologous RNA located on the replicon RNA of the present invention encodes the desired protein or peptide, and helper cells or pharmaceutical formulations containing the helper cells of the present invention are administered to a subject in need of the desired protein or peptide. In this manner, the protein or peptide may thus be produced *in vivo* in the subject. The subject may be in need of the protein or peptide because the subject has a deficiency of the protein or peptide, or because the production of the protein or peptide in the subject may impart some therapeutic effect, as a method of treatment or otherwise.

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In these examples, nm means nanometer, mL means milliliter, IU means infectious units, pfu/mL means plaque forming units/milliliter, VEE means Venezuelan Equine Encephalitis virus, EMC means Encephalomyocarditis virus, BHK means baby hamster kidney cells, HA means hemagglutinin gene, GFP means green fluorescent protein gene, N means nucleocapsid, FACS means fluorescence activated cell sorter, and IRES means internal ribosome entry site. The expression "E2 amino acid (e.g., lys, thr,

etc.) number" indicates the designated amino acid at the designated residue of the E2 protein. This convention is also used to refer to amino acids at specific residues in the E1 protein and in the E3 protein.

EXAMPLE 1

Construction of pVR2 Clone

The VEE structural protein genes (C-PE2-6K-E1) were removed from a cDNA clone (pV4031) which contained two attenuating mutations (E2 lys 209, E1 thr 272), and a duplication of the 26S subgenomic RNA promoter sequence immediately downstream from the 3'-end of the E1 glycoprotein gene followed by a multiple cloning site as described in copending Patent Application Serial No. 08/250,445, filed 27 May 1994. pV4031 plasmid DNA is digested to completion with ApaI restriction enzyme, which cuts the VEE genomic sequence at nucleotide 7505 (numbered from the 5'-end of the genome sequence). A second recognition site for this enzyme is found in the duplicate 26S subgenomic promoter. Therefore, digestion of pV4031 with ApaI produces two DNA fragments, one containing the VEE nonstructural genes and a single copy of the 26S subgenomic RNA promoter followed by a multiple cloning site, and a second smaller fragment containing a 26S subgenomic RNA promoter followed by the VEE structural genes. The large fragment is isolated and religated to produce the clone, pVR2. **Figure 1** is a graphical representation of the pV4031 clone and pVR2 clone.

EXAMPLE 2

Construction of Single RNA-Helper Plasmids

The starting materials for the helper plasmids are four full-length cDNA clones: pV3000, the virulent Trinidad donkey strain of VEE, and three clones with attenuating mutations, pV3014 (E2 lys 209, E1 thr 272), pV3519 (E2 lys 76, E2 lys 209, E1 thr 272) and pV3526 (deletion of E3 56-59, E1 ser 253), in the genetic background of Trinidad donkey strain VEE. Several different helper plasmids have been made by using unique or rare restriction sites in the full-length cDNA clones to delete portions of the nonstructural protein region. The full-

length clone is digested with one or two restriction enzymes, the larger DNA fragment is isolated and then religated to form a functional plasmid. *In vitro* RNA transcripts from these plasmids upon transfection of tissue culture cells would not encode a functional RNA replication complex, and probably also would not include an encapsidation signal. The helper constructs differ in the size of the nonstructural gene deletion. The helper constructs are designated by the attenuated mutant clone used in their construction, and by the percentage of the nonstructural region deleted. The following helper constructs were generated:

V3014Δ520-7507(93%)	V3519Δ520-7507(93%)	V3526Δ520-7505(93%)
V3014Δ520-6965(87%)	V3519Δ1687-7507(78%)	V3526Δ520-7505(93%)
V3014Δ2311-7505(70%)	V3519Δ3958-7507(47%)	
V3014Δ3958-7505(47%)	V3519Δ1955-3359(19%)	V3000Δ1955-3359(19%)
V3014Δ520-3954(46%)		
V3014Δ1955-3359(19%)		
V3014Δ1951-3359(19%)		
V3014Δ2311-3055(10%)		
V3014Δ2307-3055(10%)		

EXAMPLE 3

Construction of Double RNA-Helper Plasmids

A plasmid encoding a double helper system also is constructed, as shown in **Figure 2**. The V3014Δ520-7505(93%) single helper clone is used to construct an additional deletion of the E2 and El glycoprotein genes by digestion with HpaI restriction enzyme and subsequent ligation, resulting in deletion of the sequence between nucleotide 8494 (in the E3 gene) and nucleotide 11,230 (near the 3'-end of the El gene). *In vitro* RNA transcripts of this plasmid (shown as P21-1 in **Figure 2**), when electroporated into BHK cells with a replicon RNA are replicated and transcribed to give a mRNA encoding only the C protein of VEE.

The plasmid encoding the second member of the bipartite helper (shown as P24-3 in **Figure 2**) is constructed from the same original clone by cleavage with Tth111I restriction enzyme (at nucleotide 7544) and SpeI restriction enzyme (at nucleotide 8389) and insertion of a synthetic double-stranded

oligonucleotide with Tth111I and SpeI termini. The inserted sequence restores the downstream portion of the 26S promoter and an ATG initiation codon followed by a Ser codon, the first amino acid residue of E3. The *in vitro* RNA transcript of this plasmid when transfected into a cell with replicon RNA will produce the VEE glycoproteins. Co-transfection of both of these helper RNAs into a cell with replicon RNA results in production of infectious but replication-defective particles containing only replicon RNA. Other than the 5' and 3' ends and the 26S promoters (40 nucleotides) of these helper RNAs, the only sequence in common between the capsid and glycoprotein helper RNAs is the sequence from 8389 to 8494 (105 nucleotides).

EXAMPLE 4

Recombinant VEE Replicons Containing Heterologous Genes

The influenza HA gene, the Lassa fever virus N protein gene and the Lassa fever virus envelope glycoprotein gene, have been inserted individually into the pVR2 clone and expressed successfully in cultured BHK cells. These constructs are illustrated in the bottom third portion of **Figure 2**. Several other genes, originating from a broad range of organisms including bacteria, protozoae, and invertebrae (e.g., the *Botulinum* toxin C-fragment gene, the malaria Plasmodium CS1 gene, and the GFP gene cloned from *Aquoria victoria* jellyfish DNA by Chalfie and coworkers, *Science* **263**:802 (1994)), have also been successfully inserted into the VEE clone and expressed, as shown below in Table 1. In Table 1, blank entries indicate that the particular function has not yet been tested for that particular gene, not that tests for that function with that gene have been unsuccessful.

TABLE 1. Recombinant VEE Replicons
(Blank Indicates not tested)

	Cloning	Status		Function	
Family/Virus/Gene	Shuttle	Replicon	Expressed	Packaged	Immunogenic
Orthomyxoyviruses					
Influenza HA	+	+	+	+	+
Arenaviruses					
Lassa N	+	+	+	+	+
Lassa GPc	+	+	+	+	
Bunyaviruses					
RVFV NSM-G2-G1	+	+	+	+	
CCHF M Seg.	+	+			
CCHF M 5' half	+	+	+	+	
CCHF M 3' half	+	+	+		
SFS NSM-G1-G2	+	+			
SFS G2	+	+	+		
SFS NSm-G1	+	+	+	+	
SFS N	+	+	+		
Filoviruses					
Ebola NP	+	+	+	+	
Ebola GP	+	+	+	+	
Marburg NP	+				
Marburg GP	+				
Marburg GPt	+				
Poxviruses					
Vaccinia L1	+	+	+		
Vaccinia D8	+	+	+		
Lentiviruses					
HIV ma/ca	+	+	+	+	
HIV gp160	+	+	+		
SIV ma/ca	+	+	+	+	
SIV gp 160	+	+	+	+	
Other					
GFP	+	+	+		
Malaria CS1	+	+			
Bot-C fragment	+				

EXAMPLE 5

Detection of Heterologous Protein Expression and Packaging of Infectious Replicon Particles

Detection of protein expression in recombinant VEE replicon systems was by specific fluorescent antibody binding, except in the case of GFP, which autofluoresces when exposed to light in the range of 340-490 nm. When GFP-replicon RNA alone is electroporated into BHK cells and expression is assayed by fluorescence, greater than 95% of the cells contain active GFP. Expression levels of Lassa fever N protein in BHK cells are measured following polyacrylamide gel electrophoresis of transfected cell lysates and image analysis with NIH Image Version 1.52 on a Coomassie stained gel. Levels range from 15% to 19% of total cell protein.

GFP is packaged into infectious defective particles by co-electroporation of GFP replicon RNA and V3014Δ520-7505(93%) helper RNA, and the titer is determined by infection of BHK cells and quantitative microscopy under 400 nm light, as well as FACS analysis. The yield of replicon particles is from 2 to 6 X 10^7 per mL under these conditions. Yields using various single-helper constructs to package the Lassa fever replicon RNA ranged from 1 X 10^4 IU/mL to 8 X 10^7 IU/mL.

EXAMPLE 6

Immune Response to Replicons Packaged in Single RNA Helper System

Packaged replicons containing the Lassa fever N gene were inoculated into mice and used to induce serum antibody specific for N. The results are reported in **Figure 3**. When a low dose inoculation is used, no serum antibody specific for VEE is detected. However, there is VEE-specific antibody in the serum of a mouse inoculated with a higher dose, probably due to the replication-competent recombinants in the preparation (estimated by a plaque assay to be present at about 10^4-fold less than the titer of replicon particles). When both types of mice received a second identical dose of N replicon, both showed a significant boost in anti-N titer. *See* **Figure 3**.

EXAMPLE 7

Vaccination with Recombinant VEE Replicons in a Single RNA Helper System

Recombinant VEE replicons were constructed which expressed influenza virus HA (HA-Rep) or Lassa virus N (N-Rep). These replicons were packaged with the VEE single RNA helper system, and yields of 3 x 10^7 (HA-RepV) and 4 x 10^7 (N-RepV) infectious particles per mL were obtained for the HA and N constructs, respectively. These packaged replicons were inoculated into mice at varying doses, and the resulting immune response was monitored by immunoblots (IB) and enzyme-linked immunoassay (EIA). The mice were then challenged with influenza virus and monitored for sickness and death. The results of this experiment are presented below.

After a single immunization, all mice receiving 3 x 10^7 HA-RepV or 4 x 10^7 N-RepV seroconverted. In addition, geometric means EIA titers were significantly increased after booster immunizations. All mice receiving 3 x 10^5 HA-RepV and 4 x 10^5 N-RepV seroconverted after two immunizations. Mice receiving two doses of 3 x 10^3 HA-RepV did not seroconvert. All mice receiving two doses of 3 x 10^7 or 3 x 10^5 HA-RepV were protected against a severe influenza virus challenge. Mice receiving lower doses or control mice receiving saline were not protected.

Because these replicons were packaged with the single RNA helper, approximately 1000 plaque forming units (PFU) of VEE/ml were generated in both the HA-RepV preparations and N-RepV preparations. This resulted in the seroconversion of most of the vaccinated mice to VEE. However, as the helper RNA contained attenuating mutations (the 3014 genetic background), the regenerated VEE virus was attenuated and did not cause disease in these animals.

In order to determine if a prior immunization with one RepV interfered with subsequent immunization with a heterologous Rep V, mice were immunized first with N-RepV and subsequently with HA-RepV, using the single helper RNA system. After two inoculations of either 3 x 10^6 or 3 x 10^4 N-RepV, inoculation of two doses of 2 x 10^5 HA-RepV resulted in seroconversion in all animals to HA. No significant interference from prior immunization with N-RepV

was apparent. The subsequent challenge of these animals with virulent influenza virus demonstrated the protective immunity in all animals.

N-RepV was packaged with the single RNA helper which allowed recombination between the replicon and helper RNAs, leading to regeneration of the VEE virus. However, an immune response to HA was induced despite the fact that the animals had developed an antibody response to VEE proteins as a result of the replication-competent VEE virus in the N-RepV preparation.

EXAMPLE 8

Vaccination with Recombinant VEE Replicons Packaged in a Double RNA Helper System

As indicated above, the use of a single helper RNA to provide the structural proteins to package the recombinant replicons allows the regeneration of attentuated but fully infectious, replication-competent VEE virus by RNA recombination during co-transfection. To prevent this, a double helper RNA system is constructed in which the nucleocapsid gene is provided on one helper RNA and the glycoprotein genes are provided on a second helper RNA (see Example 3, above). Co-transfections were then carried out with the double helper system and with N-Rep and HA-Rep as above. The yields of packaged replicons, N-RepV and HA-RepV, were monitored by immunofluorescence. The presence or absence of replication-competent VEE virus were similarly monitored by immunofluorescence and by plaque assays (PFU). Media from the co-transfections were also passed into other BHK cell cultures to amplify any infectious VEE virus present; media from these flasks were subsequently assessed by plaque assay. The results are presented below in Table 2.

TABLE 2. Cotransfections with VEE replicons and double helper construct (DH)

		Immunofluorescence		Plaque assay	
		Medium from cotransfected cells			Infectious virus after amplification in BHK cells
	Date	RepU/ml[a]	VEE FFU/ml[b]	PFU/ml[c]	
N-Rep+DH	4/29/95	3x10^6	<5[d]	<50[d]	None[e]
	5/14/95	5x10^5	<5	<50	None
	7/2/95	4x10^7	<5	<50	None
	7/7/95	1X10^8	<5	<50	None
HA-Rep+DH	5/14/95	3x10^5	<5	<50	None
	7/2/95	4X10^7	<5	<50	None
	7/9/95	6x10^7	<5	<50	None

[a]Replicon units per ml, expressing heterologous gene, by IFA
[b]Focus-forming units, expressing VEE antigen by IFA
[c]Plague forming units per ml, by plaque assay
[d]Lowest detection limit of assay
[e]1 ml of media from cotransfected cells was used to infect fresh BHK cultures (75 sq cm flask), incubated for 24 and 65 hrs and monitored for infectious virus by plaque assay

The data in Table 2 demonstrate that four transfections were carried out with the double helper RNA system and N-Rep and three with this helper system and HA-Rep. Titers of packaged replicons ranged from 3 x 10^5 to 1 x 10^8. These yields are comparable to, or exceed those achieved with the single helper systems.

In no case were replication-competent VEE virus particles detected by immunofluorescence or by direct plaque assay. This is in marked contrast to results achieved with the single helper system. Replication-competent VEE virus could not be detected even after blind passage (amplification) in fresh cultures of BHK cells, which procedure can theoretically detect a single infectious unit.

Intracerebral (ic) inoculation of newborn mice is usually a more sensitive assay for infectious virus than inoculation of cell cultures. Therefore,

HA-RepV packaged with either the single or double RNA systems were inoculated at high doses into suckling mice (Table 3). The VEE glycoprotein genes used to construct both the single helper and double helper systems were obtained from the 3014 VEE construct, which is attenuated in adult mice when inoculated subcutaneously, but not in suckling mice inoculated intracerebrally. The LD_{50} of 3014 in suckling mice is 1.6 PFU.

TABLE 3. Replicon Safety Test - 1C Inoculation of Suckling Mice

Comparison of Influenza HA Replicon Packaged with Single or Double Helper

Replicon	Infectious Units Inoculated[a]	PFU Present[b]	Percent Survival
Sham Inoculated	0	0	100
HA-Double Helper	5×10^5	0	100
HA-Double Helper	5×10^7	0	100
HA-Single Helper	5×10^5	500	10
HA-Single Helper	5×10^7	50000	0

[a] Determined as focus forming units by immunofluorescence assay
[b] Determined by plaque assay on vero cells

These data indicate that no replication competent VEE virus was detected by plaque assay in the HA-RepV packaged with the double helper RNA system, but relatively high titers were found in the HA-RepV packaged with the single helper system. All suckling mice survived doses of 5×10^5 and 5×10^7 HA-RepV packaged with the double helper system, whereas none survived the high dose packaged with the single helper system, and only 1/10 survived the lower dose.

It will be shown below that a dose of 5×10^7 HA-RepV packaged with the double helper system is approximately 100 fold more than necessary to achieve an immune response in adult mice inoculated subcutaneously. Therefore, the fact that even the higher dose of packaged HA-RepV/double RNA helper is

innocuous in the most sensitive system known (suckling mice) demonstrates the high margin of safety of the double RNA helper system.

EXAMPLE 9

Immunization of Mice With HA Replicons Packaged WIth Double Helper System

N-RepV and HA-RepV produced with the double RNA helper system were inoculated into adult Balb/c mice, and the immune response to Lassa N, influenza HA, and VEE determined by enzyme-linked immunoassay (EIA). The mice were subsequently challenged with virulent influenza virus.

TABLE 4. Influenza HA Replicon packaged with Double Helper System

Immunization Schedule				Influenza Challenge[d]	
RepV	Dose[a]	Day PI[b]	VEE PFU[c]	Survive/Total	Sick/Total[e]
N-RepV DH	3×10^6	0	0		
	3×10^6	32	0		
HA-RepV DH	2×10^5	84	0		
	2×10^5	112	0	6/6	0/6

Serum EIA Titers			
Day PI	flu-HA	Lassa-N	VEE
14	<100	205	<100
84	nt	5815	<100
98	4842	nt	<100
128	20,722	nt	<100

[a] Number of infectious units as determined by immunofluorescence assay
[b] Day post inoculation
[c] Plaque forming units present in inoculum as determined by plaque assay in vero cells
[d] Mice challenged on day 136
[e] Determined visually and by observation of no significant weight loss

As the results in Table 4 indicate, no replication-competent VEE virus was detected in either the N-RepV or HA-RepV preparations by plaque assay. After a single inoculation of N-RepV on day 0, mice developed significant titers that were increased dramatically after a booster immunization on day 32. No VEE virus-specific antibody was detected.

The same mice received a subsequent inoculation of HA-RepV on day 84, and responded with significant anti-HA titers when measured on day 98. These geometric mean titers rose to 20,772 following a subsequent inoculation of the HA-RepV. The anti-HA titers demonstrate that there is no interference from prior immunization with a heterologous replicon.

After four inoculations with replicons produced from the double helper system, the mice still had no detectable EIA titer to VEE. The mice were completely protected from a challenge of virulent influenza virus when tested on day 134. This influenza virus had previously caused 50% mortality and 100% morbidity in unvaccinated mice.

EXAMPLE 10

Mutagenesis of Capsid Gene

The alphavirus nucleocapsid and glycoprotein genes of the VEE genome are normally encoded in a single open-reading frame (ORF). During translation of this ORF, the nucleocapsid cleaves itself off the growing polyprotein by virtue of an autoprotease activity. This protease activity is based on an active serine motif similar to that of chymotrypsin, which requires interaction of three distinct amino acid residues (serine, aspartate and histidine). In the VEE nucleocapsid gene, the serine, aspartate and histidine residues are located at amino acids 226, 174, and 152, respectively. Mutagenesis of these residues will compromise the protease activity of the nucleocapsid, and result in non-viable viruses as has been shown for other alphaviruses. However, in the context of the double helper system, in which the nucleocapsid gene is provided on a separate mRNA, there is no requirement for an autoprotease activity.

Experiments were performed to determine whether mutagenesis of these residues would adversely effect packaging in the context of the double helper RNA system. As indicated in Tables 5 and 6 below, site-directed mutagenesis procedures were used to alter the amino acids at three of the loci described above. These mutations were generated in a single helper RNA which encoded the nucleocapsid gene and additional (E3) sequences. Each modified construct was then co-transfected with a replicon, and the size of the nucleocapsid protein was assessed on polyacrylamide gels to determine the extent of protein self-cleavage.

Subsequently, specific combinations of mutations were examined to determine the extent of replicon packaging (as monitored by the release of infectious replicon units). In these studies, a translational termination signal (stop codon) was inserted at the end of the capsid gene.

TABLE 5. Autoprotease activity of the VEE capsid mutants

A

Capsid position	Residue	Autoprotease activity
152	H (wt)	+
	I	-
	D	-
	S	-
	O	-
	G	-
	V	-
	T	-
	A	-
	Y	-
	R	-
	F	-
	P	-

B

Capsid position	Residue	Autoprotease activity
174	D(wt)	+
	N	+
	A	+
	F	-
	L	+
	K	-
	E	+
	G	+
	T	+
	C	+
	I	-
	S	+
	H	+
	P	-
	V	-
	R	-
	Y	-

TABLE 5. continued: C

Capsid position	Residue	Autoprotease activity
228	8(wt)	+
	M	-
	Y	-
	V	-
	R	-
	N	-
	C	-
	Q	-
	G	-
	H	-
	A	-

TABLE 6. Packaging of VEE replicon by mutant VEE capsid helpers

Given 80 percentage assuming 100% packaging with the wild-type VEE capald helper containing G152; D174; and S226, nt = not tested

A

Capsid position	Residue	Packaging
152	G	<0
	F	<10
	R	<10

B

Capsid position	Residue	Packaging
174**	N	30
	O	10
	H	50
	K	30
	F	30
	W	40

**These mutants had also G mutation at position 152

C

Capsid position	Residue	Packaging
228	L	<10
	R	<10
	G	30
	N	nt

The data in Table 5 illustrate that specific mutations at each of the three loci which prevent nucleocapsid protein self-cleavage were identified. All amino acid substitutions examined at loci 152 or 226 inhibited cleavage. However, a number of different changes at locus 174 permitted cleavage to continue.

The data in Table 6 illustrate that packaging was not detectable with some permutation of mutations. However, for others, efficient packaging was observed comparable to wild-type VEE nucleocapsid helpers. Therefore, in the unlikely event that a multiple recombination event would occur among a replicon and each of the double-helper RNA system RNAs, use of such modified nucleocapsid constructs could prevent the recombinant from possessing a functional nucleocapsid gene. The modified nucleocapsid genes as described herein are functional only in the context of the double helper system.

The nucleocapsid protein altered as above provides additional assurance that recombination to produce the replication-competent virus will not occur. The altered capsid protein gene that functions in the particle assembly but not in autoproteolysis would provide helper function for production of replicon particles, but would be very unlikely to produce a viable recombinant.

The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That Which Is Claimed Is:

1. A helper cell for expressing an infectious, replication defective, alphavirus particle, comprising, in an alphavirus-permissive cell:

(a) a first helper RNA encoding *(i)* at least one alphavirus structural protein, and *(ii)* not encoding at least one other alphavirus structural protein; and

(b) a second helper RNA separate from said first helper RNA, said second helper RNA *(i)* not encoding said at least one alphavirus structural protein encoded by said first helper RNA, and *(ii)* encoding said at least one alphavirus structural protein not encoded by said first helper RNA, and with all of said alphavirus structural proteins assembling together into alphavirus particles in said cell containing said replicon RNA;

and wherein said alphavirus packaging segment is deleted from at least said first helper RNA.

2. The helper cell according to claim 1, further containing a replicon RNA;

said replicon RNA encoding said alphavirus packaging segment and an inserted heterologous RNA;

wherein said alphavirus packaging segment is deleted from at least one of said helper RNA;

and wherein said replicon RNA, said first helper RNA, and said second helper RNA are all separate molecules from one another.

3. The helper cell according to claim 1, further containing a replicon RNA;

said replicon RNA encoding said alphavirus packaging segment and an inserted heterologous RNA;

wherein said replicon RNA and said first helper RNA are separate molecules;

and wherein the molecule containing said replicon RNA further contains RNA encoding said at least one alphavirus structural protein not encoded by said first helper RNA.

4. The helper cell according to claim 1, wherein said first helper RNA encodes both said alphavirus E1 glycoprotein and said alphavirus E2 glycoprotein, and wherein said second helper RNA encodes said alphavirus capsid protein.

5. The helper cell according to Claim 1, wherein said alphavirus is selected from the group consisting of Eastern Equine Encephalitis virus, Venezuelan Equine Encephalitis virus, Everglades virus, Mucambo virus, Pixuna virus, Western Equine Encephalitis virus, Sindbis virus, Semliki Forest virus, Middelburg virus, Chikungunya virus, O'nyong-nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, and Buggy Creek virus.

6. The helper cell according to Claim 1, wherein said alphavirus comprises Venezuelan Equine Encephalitis virus.

7. The helper cell according to Claim 1, wherein said alphavirus comprises Sindbis virus.

8. The helper cell according to Claim 1, wherein said alphavirus comprises Semliki Forest virus.

9. The helper cell according to Claim 1, wherein at least one of said first helper RNA and said second helper RNA includes at least one attenuating mutation in said RNA.

10. The helper cell according to Claim 6, wherein at least one of said first helper RNA and said second helper RNA includes at least one attenuating mutation selected from the group consisting of codons at E2 amino acid position 76 which specify an attenuating amino acid, codons at E2 amino acid position 120 which specify an attenuating amino acid, codons at E2 amino acid

position 209 which specify an attenuating amino acid, codons at E1 amino acid 272 which specify an attenuating mutation, codons at E1 amino acid 81 which specify an attenuating mutation, codons at E1 amino acid 253 which specify an attenuating mutation, and the deletion of E3 amino acids 56-59.

11. The helper cell according to Claim 7, wherein said Sindbis virus comprises the S.A.AR86 strain of Sindbis virus and wherein said RNA encoding said structural proteins of S.A.AR86 virus comprise at least one attenuating mutation selected from the group consisting of codons at nsP1 amino acid position 538 which specify an attenuating amino acid, codons at E2 amino acid position 304 which specify an attenuating amino acid, codons at E2 amino acid position 314 which specify an attenuating amino acid, codons at E2 amino acid position 372 which specify an attenuating amino acid, codons at E2 amino acid position 376 which specify an attenuating amino acid, codons at nsP2 amino acid position 96 which specify an attenuating amino acid, codons at nsP2 amino acid position 372 which specify an attenuating amino acid, codons at nsP2 amino acid position 529 which specify an attenuating amino acid, codons at nsP2 amino acid position 571 which specify an attenuating amino acid, codons at nsP2 amino acid position 682 which specify an attenuating amino acid, codons at nsP2 amino acid position 804 which specify an attenuating amino acid, and codons at nsP3 amino acid position 22 which specify an attenuating amino acid.

12. The helper cell according to Claim 1, wherein said first helper RNA and said second helper RNA both include a promoter.

13. The helper cell according to Claim 2, wherein said replicon RNA includes a promoter.

14. The helper cell according to Claim 12 or 13, wherein said promoter is a Venezuelan Equine Encephalitis virus 26S subgenomic promoter.

15. The helper cell according to Claim 1, wherein said inserted heterologous RNA is selected from the group consisting of RNA encoding proteins and RNA encoding peptides.

16. A helper cell for expressing an infectious, replication defective, alphavirus particle, comprising, in an alphavirus-permissive cell:

(a) a replicon RNA encoding an alphavirus packaging sequence and an inserted heterologous RNA;

(b) a first helper RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein; and

(c) a second helper RNA encoding the alphavirus capsid protein;

so that said alphavirus E1 glycoprotein, said alphavirus E2 glycoprotein and capsid protein assemble together into alphavirus particles containing said replicon RNA therein, in said cell.

17. A helper cell for expressing an infectious, replication defective, alphavirus particle, comprising, in an alphavirus-permissive cell:

(a) a replicon RNA encoding an alphavirus packaging segment, an inserted heterologous RNA, and an alphavirus capsid protein; and

(b) a first helper RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein;

so that said alphavirus E1 glycoprotein, said alphavirus E2 glycoprotein and capsid protein assemble together into alphavirus particles containing said replicon RNA, in said cell.

18. A method of making infectious, replication defective, alphavirus particles, comprising:

transfecting an alphavirus-permissive cell according to claim 1 with a replication defective replicon RNA, said replicon RNA including said alphavirus packaging segment and an inserted heterologous RNA;

producing said alphavirus particles in said transfected cell; and then collecting said alphavirus particles from said cell.

19. The method according to Claim 18, wherein said transfecting step is carried out by electroporation.

20. A set of RNAs for expressing an infectious, replication defective alphavirus, said set comprising, in combination:

(a) a replicon RNA encoding a promoter, an inserted heterologous RNA, and wherein RNA encoding at least one alphavirus structural protein is deleted therefrom; and

(b) a first helper RNA separate from said replicon RNA, said first helper RNA encoding *in trans* said structural protein deleted from said replicon RNA and a promoter.

21. The set of RNAs according to Claim 20, further comprising a second helper RNA separate from said replicon RNA and said first helper RNA, said second helper RNA encoding *in trans* at least one structural protein, which is different from said structural protein encoded by said replicon RNA and by said first helper RNA.

22. The set of RNAs according to Claim 20, wherein said replicon RNA includes RNA encoding the alphavirus capsid protein and both the alphavirus E1 glycoprotein and alphavirus E2 glycoprotein are deleted from said replicon RNA, and wherein said first helper RNA includes RNA encoding both said alphavirus E1 glycoprotein and said alphavirus E2 glycoprotein.

23. The set of RNAs according to Claim 21, wherein said first helper RNA comprises RNA encoding a promoter and an RNA encoding both the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, said set of RNAs further comprising a second helper RNA separate from said replicon RNA and said first helper RNA, said second helper RNA encoding the alphavirus capsid protein, *in trans* from both said replicon RNA and said first helper RNA.

24. The set of RNAs according to Claim 20, wherein said replicon RNA includes a packaging sequence; and wherein said first helper RNA packaging sequence is deleted.

25. The set of RNAs according to Claim 20, wherein said alphavirus is selected from the group consisting of Eastern Equine Encephalitis virus, Venezuelan Equine Encephalitis virus, Everglades virus, Mucambo virus, Pixuna virus, Western Equine Encephalitis virus, Sindbis virus, Semliki Forest virus, Middelburg virus, Chikungunya virus, O'nyong-nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, and Buggy Creek virus.

26. The set of RNAs according to Claim 20, wherein said alphavirus comprises Venezuelan Equine Encephalitis virus.

27. The set of RNAs according to Claim 20, wherein said alphavirus comprises Sindbis virus.

28. The set of RNAs according to Claim 20, wherein said alphavirus comprises Semliki Forest virus.

29. The set of RNAs according to Claim 20, wherein said first helper RNA and said second helper RNA encoding said alphavirus structural proteins contain at least one attenuating mutation.

30. The set of RNAs according to Claim 20, wherein said first helper RNA and said second helper RNA encoding said alphavirus structural proteins contain at least two attenuating mutations.

31. The set of RNAs according to Claim 26, wherein said RNA encoding said structural proteins of Venezuelan Equine Encephalitis virus comprise at least one attenuating mutation selected from the group consisting of codons at E2 amino acid position 76 which specify an attenuating amino acid, codons at E2 amino acid position 120 which specify an attenuating amino acid, codons at E2 amino acid position 209 which specify an attenuating amino acid, codons at E1 amino acid 272 which specify an attenuating mutation, codons at E1 amino acid 81 which specify an attenuating mutation, codons at E1 amino acid 253 which specify an attenuating mutation, and the deletion of E3 amino acids 56-59.

32. The set of RNAs according to Claim 27, wherein said Sindbis virus comprises the S.A.AR86 strain of Sindbis virus and said RNA encoding said structural proteins of S.A.AR86 virus comprise at least one attenuating mutation selected from the group consisting of codons at nsP1 amino acid position 538 which specify an attenuating amino acid, codons at E2 amino acid position 304 which specify an attenuating amino acid, codons at E2 amino acid position 314 which specify an attenuating amino acid, codons at E2 amino acid position 372 which specify an attenuating amino acid, codons at E2 amino acid position 376 which specify an attenuating amino acid, codons at nsP2 amino acid position 96 which specify an attenuating amino acid, codons at nsP2 amino acid position 372 which specify an attenuating amino acid, codons at nsP2 amino acid position 529 which specify an attenuating amino acid, codons at nsP2 amino acid position 571 which specify an attenuating amino acid, codons at nsP2 amino acid position 682 which specify an attenuating amino acid, codons at nsP2 amino acid position 804 which specify an attenuating amino acid, and codons at nsP3 amino acid position 22 which specify an attenuating amino acid.

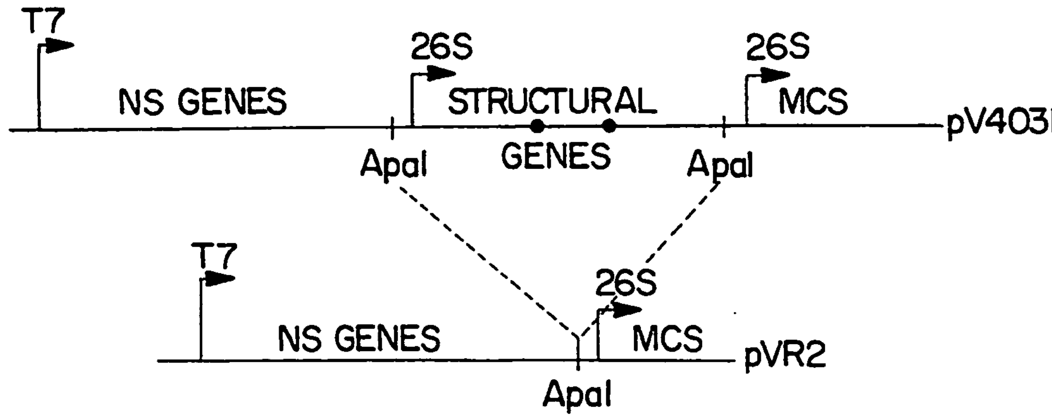
33. The set of RNAs according to Claim 26, wherein said promoter is a Venezuelan Equine Encephalitis virus 26S subgenomic promoter.

34. The set of RNAs according to Claim 20, wherein said inserted heterologous RNA is selected from the group consisting of RNA encoding proteins and RNA encoding peptides.

35. Infectious Venezuelan Equine Encephalitis virus particles containing a replicon RNA encoding a promoter, an inserted heterologous RNA, and wherein RNA encoding at least one alphavirus structural protein is deleted therefrom so that said virus particle is replication defective.

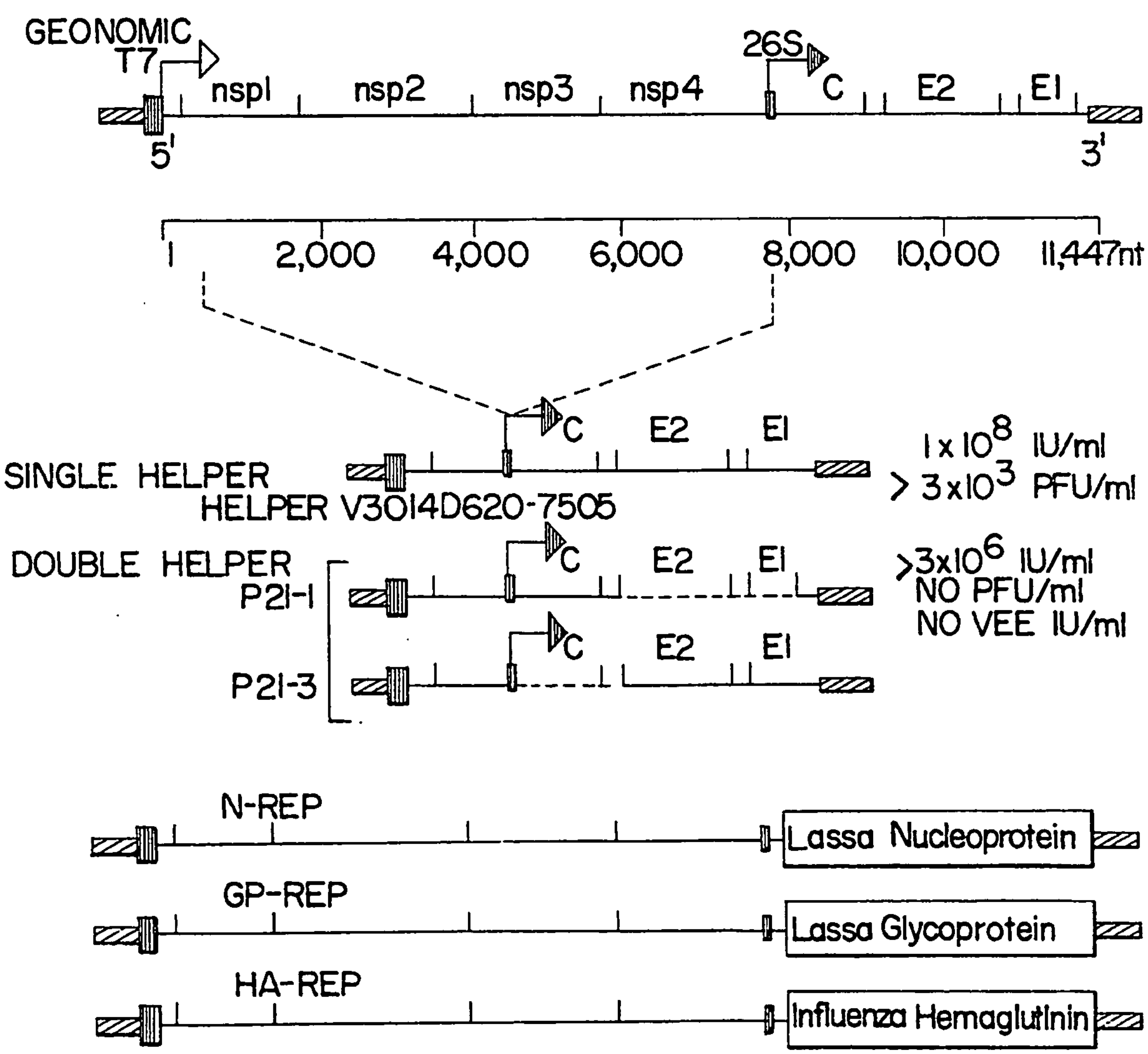
36. Infectious alphavirus particles produced by the method of Claim 18.

37. A pharmaceutical formulation comprising infectious alphavirus particles according to Claim 35 or 36 in an effective immunogenic amount in a pharmaceutically acceptable carrier.



T7
26S
26S
NS GENES
STRUCTURAL
MCS
pV4031
ApaI
GENES
ApaI
T7
26S
NS GENES
MCS
pVR2
ApaI

FIG. 1.



GEONOMIC
T7
26S
nsp1
nsp2
nsp3
nsp4
C
E2
E1
5'
3'
1
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4,000
6,000
8,000
10,000
11,447nt
C
E2
E1
1 x 10^8 IU/ml
SINGLE HELPER
> 3x10^3 PFU/ml
HELPER V3014D620-7505
DOUBLE HELPER
C
E2
E1
>3x10^6 IU/ml
P21-1
NO PFU/ml
NO VEE IU/ml
C
E2
E1
P21-3
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Lassa Nucleoprotein
GP-REP
Lassa Glycoprotein
HA-REP
Influenza Hemaglutinin

FIG. 2.

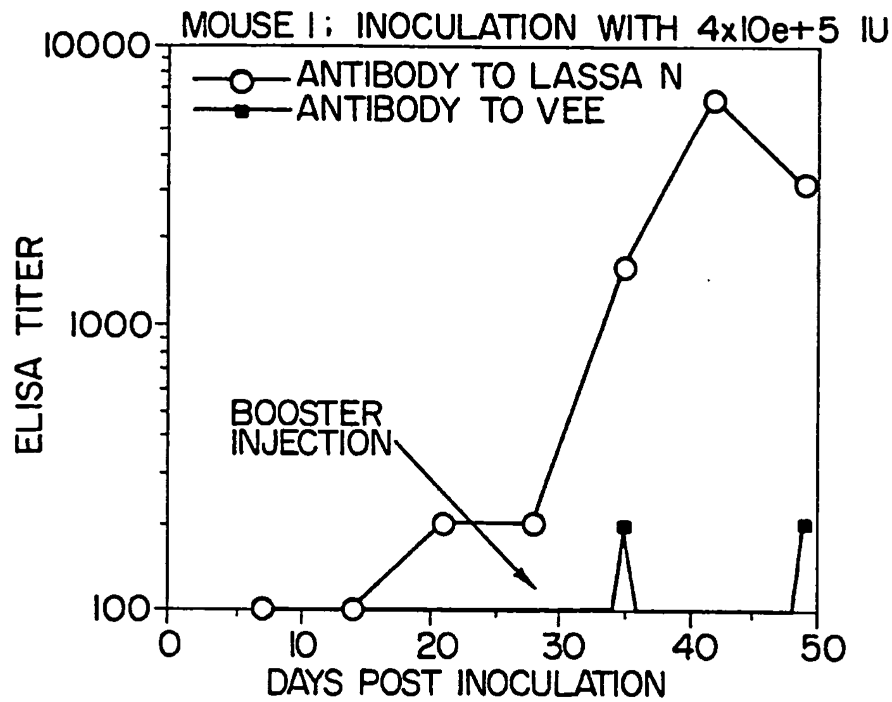
MOUSE 1; INOCULATION WITH 4x10e+5 IU
ANTIBODY TO LASSA N
ANTIBODY TO VEE
BOOSTER INJECTION
ELISA TITER
10000
1000
100
0
10
20
30
40
50
DAYS POST INOCULATION

FIG. 3A.

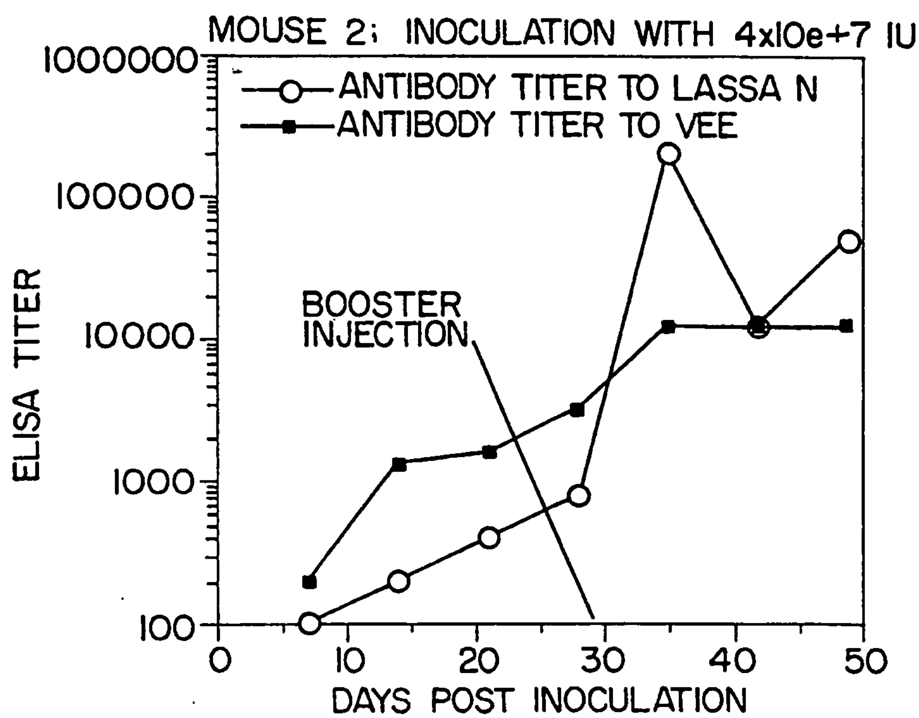
MOUSE 2; INOCULATION WITH 4x10e+7 IU
ANTIBODY TITER TO LASSA N
ANTIBODY TITER TO VEE
BOOSTER INJECTION
ELISA TITER
1000000
100000
10000
1000
100
0
10
20
30
40
50
DAYS POST INOCULATION

FIG. 3B.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/07454

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/33, 7/01; A61K 39/12, 39/193; A01N 63/00; C07H 21/04
US CL :435/69.3, 172.1, 252.3, 235.1; 424/218.1, 93.2, 93.6; 536/23.76
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.3, 172.1, 252.3, 235.1; 424/218.1, 93.2, 93.6; 536/23.76

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, USPAT, AGRICOLA, MEDLINE, DERWENT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Virology, Vol 183, issued 1991, Davis et al, "Attenuating Mutations in the E2 Glycoprotein Gene of Venezuelan Equine Encephalitis Virus: Construction of Single and Multiple Mutants in a Full-length cDNA Clone", pages 20-31, see pages 20 and 21.	1-37
Y	Journal Cell Biochemistry, Supplement O, No. 17, Part D, issued 1993, Davis et al, "A Genetically Engineered Live Virus Vaccine for Venezuelan Equine Encephalitis", page 79, see entire document.	1-37
Y	Virology, Vol 193, issued 1993, Schoepp et al, "Directed Mutagenesis of a Sindbis Virus Pathogenesis Site", pages 149-159, see pages 154-155.	1-37

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 JULY 1996	01 AUG 1996

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Virology, Vol 64, No. 9, issued September 1990, Polo et al, "Attenuating Mutations in Glycoproteins E1 and E2 of Sindbis Virus Produce a Highly Attenuated Strain when Combined in vitro", pages 4438-4444, see page 4443.	1-37
Y	Bio/Technology, Vol. 9, issued December 1991, Liljestrom et al, "A New Generation of Animal Cell Expression Vectors Based on the Semliki Forest Virus Replicon", pages 1356-1360, see page 1358.	1-37
Y	Journal of Virology, Vol 67, No. 11, issued November 1993, Bredenbeek et al, "Sindbis Virus Expression Vectors: Packaging of RNA Replicons by Using Defective Helper RNAs", pages 6439-6446, see pages 6440 and 6445.	1-37